

## A Multistate Model for the Fluorescence Response of R-Phycoerythrin

*NIST is developing a model to better understand the mechanisms by which the fluorescent molecule phycoerythrin (PE) undergoes changes in its fluorescence intensity when subjected to experimental conditions. PE emits red light when excited by a laser, but the intensity of the emission rapidly degrades, presenting a problem for scientists who wish to use this molecule for longer time-scale biomeasurements. This work is expected to help enable innovation in the biotechnology and pharmaceutical business sectors.*

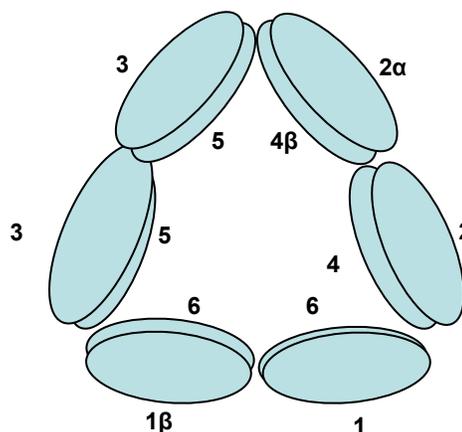
**A. Gaigalas, T. D. Gallagher, K. D. Cole, T. Singh, L. Wang, and Y. Zhang (Div. 831)**

**R**-Phycoerythrin is a widely used label in immunohistochemistry assays. The stability of its fluorescence intensity is crucial for quantitative measurement of biological molecules in living systems. Fluorescence intensity changes that occur with different time scales introduce systematic differences in measurements performed with cytometers and microscopes. Understanding the mechanism for uncontrolled fluorescence intensity variability in the phycoerythrin molecule is a first step in improvement of this vital biomeasurement tool.

Although strong fluorescence makes the R-phycoerythrin (R-PE) proteins increasingly useful in biological and clinical assays, they are subject to nonlinear effects including transitions to collective dark states and photodegradation that complicate quantitative applications. This work reports measurements of R-PE fluorescence intensity as a function of incident power, duration of illumination, and temperature. Emission intensity in the band at 570 nm is proportional to incident power for low power levels. At higher incident power, the emission at 570 nm is smaller than expected from a linear dependence on power. It is proposed that R-PE undergoes both reversible emission cessation on a millisecond time scale, attributed to transitions to a collective dark state, and irreversible photodegradation on a timescale of minutes. Singlet oxygen scavengers such as dithiothreitol and n-propylgallate have protective effects against the latter effect but not the former. Electrophoretic analysis of irradiated solutions of R-PE indicates that significant noncovalent aggregation is correlated with photodegradation.

A multistate model, based on fluorescence measurements and geometric analysis, is proposed for the fluorophores in R-PE. The phycobilin fluorophores are partitioned into three groups: the phycourobilins (PUB) absorbing at 490 nm, one group of phycoerythrobilins (PEB) absorbing at

530 nm (PEB-530), and another group of PEB absorbing at 560 nm (PEB-560). The partitioning is based on geometric analysis of the fluorophores, calculated coupling interactions, and fluorescence measurements under various conditions. Two PUB fluorophores transfer energy to the PEB-530 group which transfers energy to the PEB-560 group. The electronically excited states of PEB-530 and PEB-560 can be described as exciton states since coupling exists between all of the fluorophores within each group. The graphic shows the structure of *R*-phycoerythrin



The fluorescence emission from R-PE is due to radiative decay of the lowest excited state of the PEB-560 manifold. In addition to the radiative decay, these states undergo intersystem crossing to the triplet state, and transitions to a dark state. A relaxation pathway of the triplet state results in the production of singlet oxygen and consequent damage to the protein. The most likely place where damage occurs is where the PEB 560 groups are located. The reduction of fluorescence intensity due to prolonged irradiation was attributed to photodegradation. Frequency domain measurements show fluorescence intensity decrease with a time scale of about 6 ms that is attributed to the population of a collective dark state and subsequent non radiative relaxation to the ground state.

Both sources of fluorescence intensity reduction introduce problems in the quantitation of fluorescence intensity in biological assays. The fast variation is of importance in flow cytometers and scanning instruments. The slow variation (photodegradation) is more relevant to imaging with long exposures under constant illumination, such as microscope imaging applications.